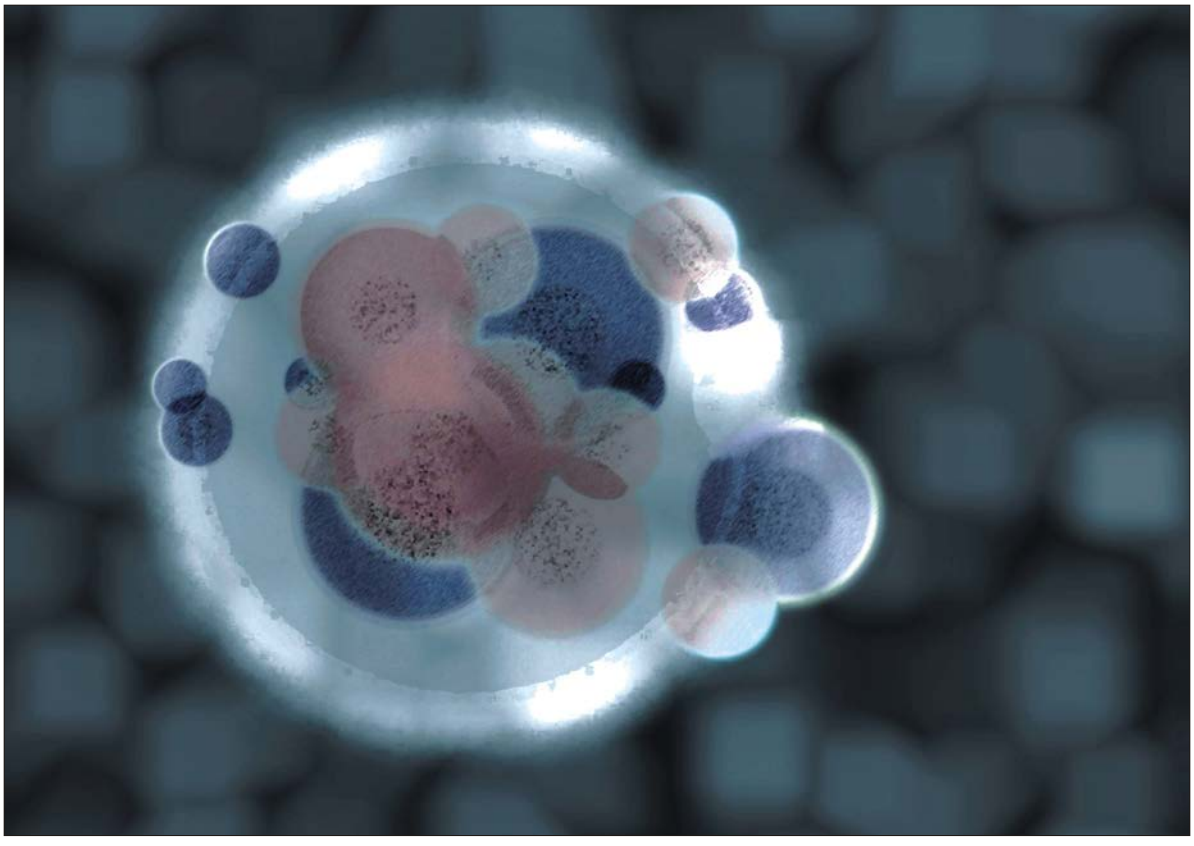

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Effect of Three Sewage Isolated Bacteriophages on the Multidrug Resistant Pathogenic Bacteria

[1]Nafiseh Sadat Naghavi, [1]Mahdieh Golgoljam and [2]Mojgan Akbari
[1]Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran
[2]Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran

Abstract: Bacteriophages have been proposed as specific alternative for chemotherapy against multidrug resistant bacterial infections. The aim of the present study was the isolation and purification of bacteriophages from sewage, which were effective against multidrug resistant bacterial isolates and detection of their morphological characteristics. Sewage and clinical samples were used for the isolation of antibiotic resistant pathogenic bacteria. The bacteriophages were isolated and purified from sewage samples by overlay cultures of the isolated bacteria after centrifugation and filtration procedures. Three isolates of the most multidrug resistant *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* selected for bacteriophage isolation. Most of the isolates were resistant to different classes of antibiotics in the aminoglycosides, cephalosporins, macrolides, tetracyclines and β-lactam antibiotic groups. The isolated bacteriophges showed different plaque morphologies on the bacterial cultures. The three purified bacteriophages had icosahedral or hexagonal heads. Long tails observed in *Escherichia coli* bacteriophages, with the total length of about 170 nm for the phages particles; short tails observed in *Klebsiella pneumonia* phages particles, with the approximate total lengths of 150 nm; and short tails with the approximate 35 nm total particles length observed in *Pseudomonas aeruginosa* bacteriophages.

Key words: Bacteriophage, sewage, pathogenic bacteria, multidrug resistance

INTRODUCTION

Because of the progressive resistance of pathogenic bacteria to ordinary antibiotics, bacteriophage (phage) therapy has been proposed as an effective supplement for chemotherapy against multidrug resistant bacterial infections (Knipe *et al*., 2001). Lytic phages had been recognized for remarkable antibacterial activities which are comparable to the most effective antibiotics (Meladze *et al*., 1982; Kochetkova *et al*., 1989). However, some more advantages have been reported for the usage of bacteriophages in the therapeutic procedures. Bacteriophages are very specific and therefore have less effect on normal microflora of the body. They replicate in the site of infection and have fewer side effects on the body cells. Also selecting of the new bacteriophges for resistant bacteria is a relative rapid procedure (Sulakvelidze *et al*., 2001).

Chan and Abedon (2012) reported that the usage of cocktails which consist of bacteriophages combines is more effective than individual bacteriophages for treatment of multidrug resistant bacterial infections.

Bacteriophages also have been used for treatment of animal and plant infections. For example, Wills *et al.* (2005) used specific phages for controlling *Staphylococcus aureus* abscesses in rabbits. Also, several plant pathogenic bacteria such as *Erwinia* and *Xanthomonas* have been reported that have been eliminated using specific bacteriophages (Boule *et al*., 2011).

The aim of present study was detection of the effects of the sewage isolated bacteriophages on multidrug resistant pathogenic bacteria isolated from clinical and sewage samples.

MATERIALS AND METHODS

Samples and isolation of the bacteria: Two groups of samples consist of sewage and clinical samples were used for the isolation of antibiotic resistant bacteria. After serial dilutions prepared from sewage, each dilution inoculated to Nutrient broth (NB) and Brain heart infusion broth (BHI). The culture media incubated at 37°C for 24 h. After growth, the bacteria were translated to blood agar and nutrient agar media for purification of the isolated

Corresponding Author: Nafiseh Sadat Naghavi, Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran, PO Box: 8451731167 Fax: 00983117420136

bacteria. The clinical specimens were achieved from stool, urine and skin wounds of the hospitalized symptomatic patients. The purified isolates were detected according to morphological and biochemical characteristics (Brooks *et al*., 2010; Mahon *et al*., 2010).

Antibiotic resistance analysis: The kirby-bauer method (Mahon *et al*., 2010) used for selection of the isolates which were resistant to different classes of antibiotics.

Isolation of bacteriophages: The amount of 25 mL of every sewage sample was centrifuged in 280 g for 10 min. The supernatant was filtered using 0.22 μm filters. The filtrates transferred to 100 mL flasks and 10 mL Tripticase Soy Broth (TSB) media with double concentration were added to them. The cultures inoculated with 1.5×10^8 bacterial cells and incubated at 37°C for 24 h. The cultures were centrifuged in 280 g for 10 min and filtrated using 0.22 μm filters. A solution consists of chloroform (0.2 mL) and 1.5×10^8 cells of the isolated bacteria were added to each filtrate and incubated at 37°C for 24 h. Then the filtrates were centrifuged 10 min in 280 g for two times. The above procedure repeated 7 times. A serial dilution (10^{-1}-10^{-10}) was prepared for each filtrate. Each dilution inoculated to a nutrient broth medium containing 0.7% agar and a final concentration of 0.01 M calcium chloride for increasing the efficiency of phage infection (Foddai *et al*., 2009). An inoculum of 1.5×10^8 cells of each isolated bacterium was inoculated to the above media and overlaid in the petri dishes. The plaques were detected and counted after 24 h incubation at 37°C.

Purification of the isolated bacteriophages: Individual plaques were cut and transferred to double concentrated TSB media. The media were inoculated with 1.5×10^8 cells of each isolated bacterium. The cultures were centrifuged 10 min in 280 g and filtered using 0.22 μm filters, after 24 h incubation at 37°C. The purified bacteriophages were treated on all of the isolated bacteria. The purified bacteriophages were peaked from the cultures by cutting the plaques and transferred to SM buffer containing per liter: 5.8 g NaCl, 2 g $MgSO_4.H_2O$, 50 mL Tris-HCl (1M, pH 7.5) and 2% gelatin (Matsuzaki *et al*., 2003). The buffer was supplemented with 20% chloroform for better preservation of bacteriophage particles (Sambrook and Russell, 2001).

Transmission electron microscopy (TEM): The purified bacteriophaged were stained with radioactive uranyl acetate and observed with TEM (Philips CM10).

RESULTS

The isolated bacteria: Three species of antibiotic resistant bacteria isolated and identified from clinical and sewage samples. The isolated strains were detected as three *Escherichia coli*, two *Klebsiella pneumonia* and two *Pseudomonas aeruginosa* isolates. The morphological and biochemical charachteristics of the isolated bacteria is shown in Table 1. Also, Table 2 represents the resistance features of the isolated bacteria to different groups of antibiotics.

Isolation and purification of the bacteriophages: The most multidrug resistant isolate of each species, i.e the isolate number 3 for *Escherichia coli*, the isolate number 6 for *Klebsiella pneumonia* and the isolate number 8 for *Pseudomonas aeruginosa* (Table 2), were selected for the procedure of bacteriophage isolation and purification. The morphology of plaques on culture media for three isolates is shown in Fig. 1. The simple plaques observed on *Escherichia coli* and *Pseudomonas aeruginosa* cultures, although the plaques on *Klebsiella pneumonia* culture had central dots. The number of plaque forming units (PFU mL^{-1}) in the bacterial cultures is demonstrated in Table 3. The results showed significant plaque counts in all bacterial cultures with the most count for *Escherichia coli* bacteriophage (4952×10^7 PFU mL^{-1}).

Morphology of bacteriophages particle in TEM: As shown in Fig. 2, all three bacteriophges had a complex structure of an icosahedral or hexagonal head and a tail. A long tail was observed in *Escherichia coli* bacteriophages, with the total length of about 170 nm for the phage particle. The total length of the *Klebsiella pneumonia* phage particle was about 150 nm, with a short tail. Also, *Pseudomonas aeruginosa* bacteriophage had short tail and the total length of the phage particle was about 35 nm.

DISCUSSION

In the present study, we reported the isolation of a specific phage against an isolate of multidrug resistant *Escherichia coli*. TEM micrograph of the phage showed an icosahedra head and a long tail. The morphological appearance of the phage is similar to the lambda which previously reported (Hershey and Dove, 1983). Also, Begum *et al*. (2010) isolated the phage IMM-001 from surface water samples, which showed similar morphology to lambda and was effective against enterotoxigenic

Table 1: The morphological and biochemical characteristics of the isolated bacteria

Isolate number characteristics	Clinical	Clinical	Clinical	Sewage	Sewage	Clinical	Clinical	Clinical
Morphology and gram staining	Gram-negative coccobacilli	Gram-negative coccobacilli	Gram-negative coccobacilli	Gram-negative coccobacilli	Gram-negative coccobacilli	Gram-negative coccobacilli	Gram-negative rods	Gram-negative rods
Pigment on mueller hinton agar	-	-	-	-	-	-	Green	Green
Indole production	+	+	+	-	-	-	-	
Colony on eosin methylen blue agar	Metallic green sheen (lactose fermentative)	Metallic green sheen (lactose fermentative)	Metallic green sheen (lactose fermentative)	Pink (lactose fermentative)	Pink (lactose fermentative)	Pink (lactose fermentative)	*	*
Methyl red reaction	+	+	+	-	-	-	-	-
Voges-proskauer reaction	-	-	-	+	+	+	-	-
Citrate utilization	-	-	-	+	+	+	+	+
Urease	-	-	-	+	+	+	+	+
SH_2 production	-	-	-	-	-	-	-	-
Motility	+	+	+	-	-	-	+	+
Catalase	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	+	+
Reaction on TSI	A/A Gas	A/A Gas	A/A Gas	A/A Gas	A/A Gas	A/A Gas	K/K	K/K
Oxidative/fermentative	Fermentative	Fermentative	Fermentative	Fermentative	Fermentative	Fermentative	Oxidative	Oxidative
Mannitol fermentation	+	+	+	+	+	+	*	*
Xylose fermentation	+	+	+	+	+	+	*	*
Sorbitol fermentation	+	+	+	+	+	+	*	*
Saccharose fermentation	+	+	+	+	+	+	*	*
Arabinose fermentation	+	+	+	+	+	+	*	*
Identified species	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*	*Klebsiella pneumonia*	*Klebsiella pneumonia*	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*

+: Positive reaction, -: Negative reaction, *: Not valuable to perform

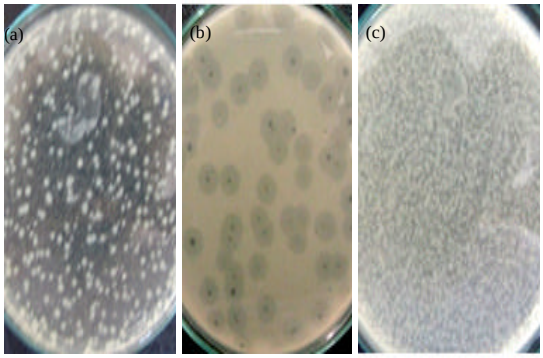

Fig. 1(a-c): Morphology of the purified plaques on the culture media, (a): *Escherichia coli* culture with simple plaques, (b): *Klebsiella pneumonia* culture with central dots and (c): *Pseudomonas aeruginosa* culture with simple plaques

Table 2: Antibiotic resistance features of the isolated bacteria

Bacterial isolate	Resistant to the antibiotics*
Escherichia coli (isolate 1)	Amk, Tbm, Clx, Cfix, Ctx, Erm, Tet, Amx, Gent, Clox, Pcn, Pipc.
Escherichia coli (isolate 2)	Clx, Cfix, Ctx, Tet, Clox, Pcn, Erm, Amx.
Escherichia coli (isolate 3)	Gent, Tbm, Clx, Cfix, Cfx, Ctz, Ctx, Erm, Tet, Amx, Clox, Pcn.
Klebsiella pneumonia (isolate 4)	Erm, Clox, Pcn.
Klebsiella pneumonia (isolate 5)	Erm, Clox.
Klebsiella pneumonia (isolate 6)	Ctz, Ctx, Amx, Clox.
Pseudomonas aeruginosa (isolate 7)	Clx, Cfix, Amx, Clox, Pcn
Pseudomonas aeruginosa (isolate 8)	Gent, Tbm, Clx, Cfix, Ctx, Erm, Tet, Amx, Clox, Pcn.

*Aminoglycosides (Amk: Amikacin, Gent:Gentamicin, Tbm: Tobramycin) cephalosporins (Clx: Cefalexin, Cfix: Cefixime, Cfx: Cefotaxime, Ctz: Ceftazidime, Ctx: Ceftriaxone), macrolides (Erm: Erythromycin), Tet: Tetracyclines and β-lactams (Amx: Amoxicillin, Clox: Cloxacillin, Pcn: Penicillin, Pipc: Piperacillin)

Table 3: Number of plaque forming units (PFU mL^{-1}) in each bacterial isolate culture

Bacterial isolate	PFU mL^{-1}
Escherichia coli (isolate 3)	4952×10^{7}
Klebsiella pneumonia (isolate 6)	247×10^{6}
Pseudomonas aeruginosa (isolate 8)	1150×10^{7}

Escherichia coli. They concluded that the isolated phage had different genetically characteristics from lambda, but additional gene screening was needed for exact differentiation of the phage from lambda.

The morphology of the *Klebsiella pneumonia* phage was similar to Phage SS, which had been isolated and detected by Chhibber *et al.* (2008). This phage showed an icosahedra head and a short tail.

The isolated *Pseudomonas aeruginosa* phage showed the specific characteristics of phage JG004 which had been isolated previously from

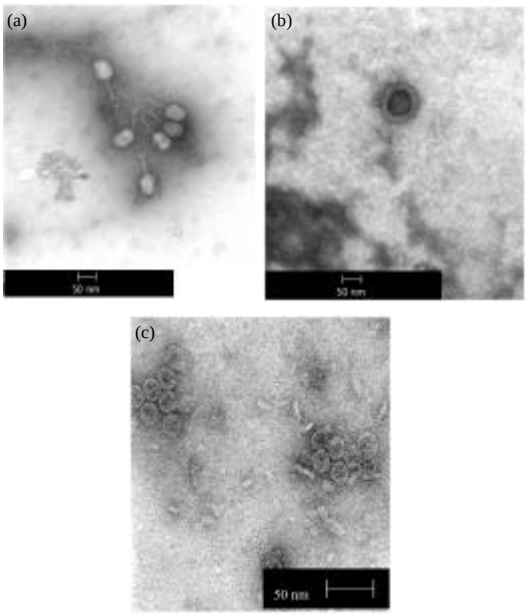

Fig. 2(a-c): Transmission electron micrographs of the isolated bacteriophages are shown, (a): *Escherichia coli* bacteriophages with icosahedral heads and long tails. The total length of the particle is about 170 nm, (b): *Klebsiella pneumonia* bacteriophage with icosahedral head and short tail, The total length of the particle is about 150 nm and (c): *Pseudomonas aeruginosa* bacteriophages with hexagonal heads and short tails, The total length of the particle is about 35 nm

Pseudomonas aeruginosa with a hexagonal head (Garbe *et al.*, 2011), but the length of the present isolated phage is obviously shorter.

The resistant of bacteria to novel antibiotics is the most important challenge in the treatment of infections. For example 80% of nosocomial infections caused by multidrug resistant strains of *Klebsiella pneumonia* (Chhibber *et al.*, 2008). Phage therapy is proposed as an effective and specific supplement therapy especially in progressing countries.

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